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ORIGINAL ARTICLE

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Mechanisms of arsenic hyperaccumulation in *Pteris* species: root As influx and translocation

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Abstract Several species of fern from the *Pteris* genus are able to accumulate extremely high concentrations of arsenic (As) in the fronds. We have conducted short-term unidirectional As influx and translocation experiments with ^{73}As-radiolabeled arsenate, and found that the concentration-dependent influx of arsenate into roots was significantly larger in two of these As-hyperaccumulating species, *Pteris vittata* (L.) and *Pteris cretica* cv. Mayii (L.), than in *Nephrolepis exaltata* (L.), a non-accumulating fern. The arsenate influx could be described by Michaelis-Menten kinetics and the kinetic parameter K_m was found to be lower in the *Pteris* species, indicating higher affinity of the transport protein for arsenate. Quantitative analysis of kinetic parameters showed that phosphate inhibited arsenate influx in a directly competitive manner, consistent with the hypothesis that arsenate enters plant roots on a phosphate-transport protein. The significantly augmented translocation of arsenic to the shoots that was seen in these As hyperaccumulator species is proposed to be due to a combination of the increased root influx and also decreased sequestration of As in the roots, as a larger fraction of As could be extracted from roots of the *Pteris* species than from roots of *N. exaltata*. This leaves a larger pool of mobile As available for translocation to the shoot, probably predominantly as arsenite.

Keywords Arsenate · Arsenite · Membrane transport · Phosphate · Phytoremediation · *Pteris*

Abbreviations As^V: Arsenate · As^{III}: Arsenite · K_m: Michaelis-Menten constant · P_i: Phosphate · V_{max}: Maximum rate of an enzyme-catalyzed reaction

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Introduction

The first plant species identified to hyperaccumulate arsenic (As), *Pteris vittata* L., was reported 3 years ago by Ma et al. (2001). *P. vittata* can accumulate As in its fronds to values up to 21 g kg^{-1} after growing for 6 weeks in soil containing 0.5 g kg^{-1} As. This discovery opens up the possibility for phytoremediation of As-contaminated soil and groundwater.

The adverse health effects of As are well documented (National Research Council 1977, 1999). Arsenic occurs naturally in the environment, but has also been anthropogenically elevated. For example, As pesticides were commonly used on agricultural land in the USA prior to 1968 (Ganje and Rains 1982). Arsenic, predominantly from natural sources, has also been found to be contaminating groundwater in parts of the Indian sub-continent and South-East Asia, and in the USA the US Environmental Protection Agency has recently lowered the Maximum Contaminant Level for drinking water from 50 to 10 μg l^{-1}, effective in 2006.

Therefore, the study of the mechanisms responsible for As hyperaccumulation is not only interesting from a scientific standpoint—how (and why?) does a plant accumulate and tolerate such huge quantities of a toxic substance—but also has potential implications in the development of phytoremediation technologies.

A better understanding of mechanisms of As hyperaccumulation would assist the development of genetically engineered plants, either from the *Pteris* genus or other species, with improved features for phytoremediation.

Since its discovery, much study has been directed towards the accumulation and speciation of As in the fronds of *P. vittata* (e.g. Tu et al. 2002). Work on hyperaccumulation of heavy metals, e.g. Cd and Zn, in other accumulator plants has shown that root uptake and transport mechanisms can be important in hyperaccumulation (Lasat et al. 1996, 1998). The route of arsenate (As^V) uptake by plant roots, which has been studied in non-accumulating species, is thought to be via a phosphate (P_i)-uptake system (Meharg and Macnair 1992) as As^V is a P_i analog. In *P. vittata*, Wang et al. (2002) have recently shown that P_i inhibited the removal of As^V from solution, although they were unable to rule out indirect effects of P_i, or to determine if the inhibition was competitive, which would be consistent with both species being transported via the same system.

Arsenic enters *P. vittata* in the root and accumulates in the shoot, but very little is known about the fate of As in the plant between entry and final destination, and what differences in form or route may result in As hyperaccumulation in shoots of *P. vittata*.

Since the discovery of the As-hyperaccumulating ability of *P. vittata*, several other fern species, mostly in the *Pteris* genus, have also been found to hyperaccumulate As (Zhao et al. 2002; Meharg 2003). We chose to study the uptake and distribution of As in two As-hyperaccumulating *Pteris* species (*P. vittata* and *P. cretica* cv. Mayii L.) and to compare these with a non-accumulating fern species, *Nephrolepis exaltata* (Meharg 2003).

We have used the radioactive tracer technique, the sensitivity of which permits the use of both small samples and short influx times. This allows the more accurate determination of unidirectional influx, rather than net uptake (the combined result of influx and efflux), which is measured over longer time periods by other methods. The aims of our work were: (i) to compare the rates of As^V uptake into roots of As-hyperaccumulating and non-accumulating plant species and to determine the concentration-dependent kinetic parameters for this uptake; (ii) to quantify the effect of P_i on As^V uptake as well as the kinetic parameters for this interaction, in order to address the question of whether P_i inhibition of As^V uptake is due to direct competition; (iii) to determine how quickly As^V is reduced to arsenite (As^{III}) in the roots and if there is greater reduction in the As hyperaccumulator species; (iv) to examine differences in As translocation between As-hyperaccumulating and non-accumulating species, and also if there is any effect of P_i on As translocation, which would suggest that long-distance As transport involves one or more P_i-transport pathways; and (v) to study the As species present in the shoots of hyperaccumulator species, and speciation changes over time.

Materials and methods

Plant material

Two *Pteris* species, *P. vittata* L. and *P. cretica* L. cv. Mayii, which have both been shown to hyperaccumulate As (Ma et al. 2001; Meharg 2003), were studied and were compared with *Nephrolepis exaltata* L., a fern species which has been shown not to accumulate As (Meharg 2003). Fern seedlings were obtained from Milestone Agriculture Inc. (Apopka, FL, USA) 16 weeks after spore germination and were approximately 7 cm tall. The roots were washed in tap water to remove the peat/perlite potting mix. The seedlings were transferred to a hydroponic system, in which plants were held in slits cut in a combined layer of nylon mesh and black plastic (to minimize illumination of the growth solution). The mesh layer was supported by a plastic grid over 30 cm×45 cm trays containing 10 l of aerated nutrient solution. The composition of the nutrient solution was in mM: K 0.3 (as KNO_3, KH_2PO_4, and KCl); Ca 0.2 (as $CaCl_2$); Mg 0.2 (as $MgSO_4$); S, 0.2 (as $MgSO_4$); N 0.7 [as NH_4NO_3, $Ca(NO_3)_2$, and KNO_3]; P 0.2 (as KH_2PO_4); and in μM, Mn 0.5 (as $MnSO_4$); Zn, 0.5 (as $ZnSO_4$), Cu 0.5 (as $CuSO_4$); Mo 0.1 (as Na_2MoO_4); Ni 0.1 (as $NiSO_4$); and Fe, 2.0 (as Fe-EDDHA). Seedlings were grown for 4–6 weeks, with a light/dark regime of 16/8 h at 25°C/20°C, under a light intensity of approximately 500 μmol photons m^{-2} s^{-1}, by which time each plant had five to six fronds and was approximately 10 cm tall. All plants used in these experiments were of similar size, with approximate fresh weights of 2.4 g for roots and 2.6 g for shoots.

Radiotracer ($^{73}As^V$) uptake experiments

Twelve hours before the experiment, plants were transferred to 0.1 mM $CaCl_2$ solution. Intact plants were placed in individual Plexiglas wells of an ion-uptake apparatus (having 20 uptake wells) filled with fresh 0.1 mM $CaCl_2$ solution. One hour after the plants were transferred to uptake wells, experiments were initiated by replacing the $CaCl_2$ solution with 75 ml uptake solution, which contained 0.1 mM $CaCl_2$ and 555 kBq l^{-1} of ^{73}As-labeled As^V in all experiments, plus the concentrations of non-radioactive As^V (as Na_2HAsO_4) and, where appropriate, P_i (as KH_2PO_4/K_2HPO_4 at pH 6.5), as outlined below. ^{73}As was in the form of As^V (verified using an As speciation column, see below), with a specific activity of >37 MBq ml^{-1} and a half-life of 80.3 days (obtained from Oak Ridge National Laboratory, http://www.ornl.gov/isotopes/catalog.htm). Therefore, the uptake of As^{III} was not studied in these experiments. After the uptake period, uptake solution was withdrawn by vacuum and roots were rinsed in ice-cold deionized (DI) water for 30 s, followed by 5 min in ice-cold desorption solution (0.1 mM $CaCl_2$ and 1 mM

KH_2PO_4/K_2HPO_4 at pH 6.5), to remove any adsorbed radioisotope. Ice-cold rinsing solutions were used to minimize any enzyme-mediated membrane transport during the rinse and desorption periods. A preliminary experiment comparing cold (4°C) and room-temperature (25°C) rinsing solutions showed that this treatment did not cause a 'cold-shock', in which ions may be released from root cells, data not shown. The plants were blotted, separated into roots and shoots, weighed and the ^{73}As activity determined using a Gamma Counter (Wallac 1480 Wizard 3'' Gamma Counter; Perkin Elmer, Norwalk, CT, USA).

Uptake and distribution of As

The uptake solution initially contained 2.67 μM (200 μg l^{-1}) ^{73}As-labeled As^V and uptake times were 6 or 24 h. Results are presented as quantity of As (μg) in each 'compartment' (solution, roots, or shoots) at the end of the experiment.

Concentration-dependent kinetics of As influx and effect of P_i on As influx

The effect of P_i on As^V uptake by the three fern species was studied in an initial experiment using an uptake solution containing 2.67 μM ^{73}As-labeled As^V and 0, 50, 100, 250 or 500 μM P_i, with an influx time of 60 min. In the concentration-dependent As-influx experiments, uptake solution contained As^V at either 0.1, 0.5, 1.5, 2.5, 5 or 7.5 μM ^{73}As-labeled As^V, either without P_i, or with 5 or 30 μM P_i (chosen to give, respectively, an equimolar concentration of P_i and As^V, and to give a significant, but not total inhibition of As^V uptake, see Results). The influx time was 30 min. The rates of As^V influx (nmol As g FW^{-1} h^{-1}) were calculated and the data were transformed using the Hanes-Woolf linear transformation (Segel 1976). The K_m and V_{max} values calculated from this plot were very similar to those independently derived from models generated from the Michaelis-Menten equation, in which K_m and V_{max} were varied to give the best fit to the data points (Roy et al. 2003). Values presented are from the Hanes-Woolf transformation and fitted lines in Figs. 2 and 3 are Michaelis-Menten models, using these calculated values.

Arsenic speciation in roots and shoots

In the speciation experiments, uptake solution contained 2.67 μM ^{73}As-labeled As^V, no P_i, and uptake times were 1, 5, 12 or 24 h. *P. cretica* and *N. exaltata* were studied after exposure to As^V for all these times, but *P. vittata* was only studied after 1 and 5 h. A sample of approximately 2 g of blotted root or shoot material was taken and the total ^{73}As activity of the sample was determined by gamma counting. The sample was then coarsely chopped using scissors and ground using an ice-cold mortar and pestle, with 10 ml ice-cold 95% ethanol, followed by further grindings with 5 ml ice-cold 95% ethanol, 10 ml and then 5 ml ice-cold DI water. Each sample took approximately 6 min to extract. All extracts from a sample, plus the remaining tissue from the mortar, were transferred to a 50-ml centrifuge tube and centrifuged for 15 min at 800×*g*. The ^{73}As activity in the extract (termed 'extractable arsenic') was determined by counting a 15-ml sample of the centrifuged extract. This 15-ml extract sample was then passed through an As speciation column (Dr. X. Meng, Department of Civil, Environmental, Ocean Engineering, Stevens Institute of Technology, NJ, USA), which retains As^V, but not As^{III} (or organic As species). This was followed by a wash of 5 ml ice-cold DI water through the column to elute any remaining As^{III}. Only one 5-ml water rinse was required as negligible As^{III} could be eluted from the column by a second rinse. All tissue and extract samples were kept on ice or in a refrigerator when not in the centrifuge or gamma counter. The entire procedure, from harvesting tissue to counting eluted As^{III} samples, took approximately 75 min.

Arsenic translocation study

In the translocation experiment, uptake solution contained 2.67 μM ^{73}As-labeled As^V and either 0, 5 or 30 μM P_i. The uptake time was 24 h.

Data analysis

Values in both text and figures are quoted as means ± SE (standard error of the mean). Statistical significance (at 95% confidence) was tested using either Student's *t*-test or one-way, two-way or factorial ANOVA, as appropriate, and numbers of replicates are included in figure legends.

Results

When incubated in ^{73}As-labeled As^V solution for 6 h, roots of the As hyperaccumulator *Pteris cretica* cv. Mayii took up a much greater amount of As than the non-accumulator *Nephrolepis exaltata* (Fig. 1). In *P. cretica*, after 6 h approximately 60% of the original solution As was in the plant (roots and shoots combined), of which 50% had already been translocated to the shoots. In comparison, after 6 h *N. exaltata* had only accumulated 12% of the As that was originally in the solution. In addition to this faster root uptake of As^V, *P. cretica* also exhibited a faster translocation of As to the shoot. After 24 h, not only had *P. cretica* completely depleted the solution of As, but 67% of the As in the plant had been translocated to the shoot. In contrast, in *N. exaltata*, even after 24 h of accumulation, by which time the roots

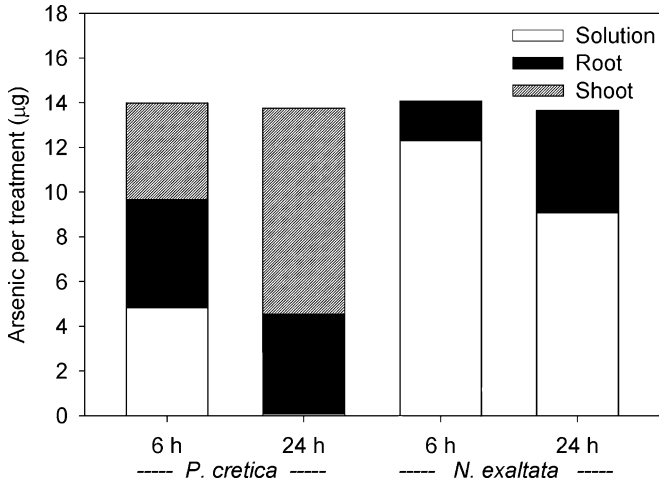

Fig. 1 Distribution of As among solution, roots and shoots of *Pteris cretica* and *Nephrolepis exaltata* after 6 and 24 h in ^{73}As-labelled As^V uptake solution, which initially contained 15 μg As^V (2.7 μM). Arsenic lost during desorption was not measured. Tissue concentrations (in nmol g FW^{-1}) in *P. cretica* were: 29 (root), 23 (shoot) at 6 h; 19 (root), 38 (shoot) at 24 h and in *N. exaltata* were: 17 (root), 0.2 (shoot) at 6 h; 45 (root), 0.7 (shoot) at 24 h. Data points represent means of 5 replicates, except for *N. exaltata* at 6 h where $n=4$

had accumulated an appreciable amount of As (31% of that originally in the solution), less than 3% of the As in the plant had been translocated to the shoot.

Influx of As^V into the root

The As^V root influx was significantly larger ($P<0.001$) in the two *Pteris* species than in *N. exaltata* over the As^V

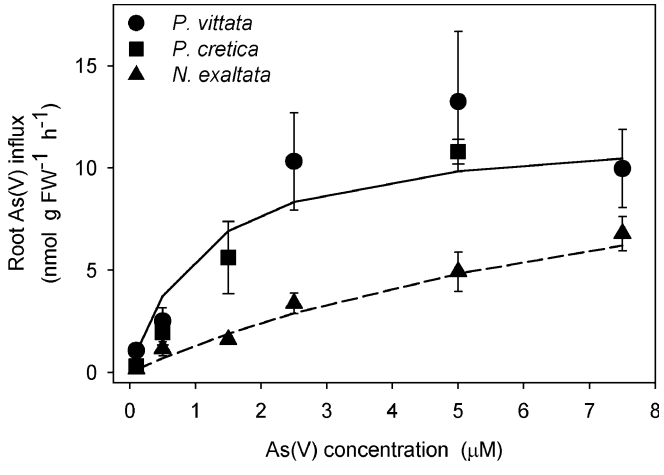

Fig. 2 Concentration-dependent influx of ^{73}As-labelled As^V into roots of *P. vittata*, *P. cretica* and *N. exaltata*. The influx time was 30 min. Root tissue concentrations (in nmol g FW^{-1}) were 6.6 for *P. vittata*, 5.4 for *P. cretica* and 2.5 for *N. exaltata* at 5 μM As^V. Data points and error bars represent means ± SE of 3 or 4 replicates and some error bars are smaller than the symbol. Lines are models generated by inserting the derived kinetic parameters into the Michaelis-Menten equation: *solid line*, *P. vittata*; *dashed line*, *N. exaltata* (model has not been fitted to *P. cretica* data)

Table 1 Kinetic parameters for concentration-dependent influx of ^{73}As-labelled As^V into intact roots of *Pteris vittata* and *Nephrolepis exaltata* with and without phosphate (P_i). Influx time was 30 min and errors are ± SE

	V_{max} (nmol g $FW^{-1}h^{-1}$)		K_m (μM)	
	0 μM P_i	30 μM P_i	0 μM P_i	30 μM P_i
P. vittata	12.0 ± 1.8	9.5 ± 0.8	1.1 ± 0.3	6.8 ± 1.9
N. exaltata	14.4 ± 2.0	9.7 ± 2.6	9.9 ± 2.0	19.9 ± 3.3

concentration range used in the kinetic study (Fig. 2). The short-term unidirectional influx of As^V into roots was concentration-dependent in all three species. The influx followed Michaelis-Menten kinetics, as the data transformed into a linear plot, the Hanes-Woolf plot, when concentration/rate versus concentration was plotted, the slope of which gives $1/V_{max}$ and y-intercept gives K_m/V_{max} (Segel 1976). The r^2 values for these linear plots (not shown) were 0.85 for *P. vittata* and 0.70 for *N. exaltata*.

There was no significant difference in V_{max} values between *P. vittata* and *N. exaltata*, whereas the species had significantly different K_m values ($P=0.02$): *N. exaltata* had a K_m value nine times greater than that of *P. vittata* (Table 1). High variability in the *P. cretica* influx data precluded quantitative analysis; therefore *P. vittata* was subsequently used to represent the As hyperaccumulator species.

Phosphate inhibited As^V uptake into the roots of all three fern species studied. In an initial experiment with 2.7 μM As^V in the uptake solution, 50 μM P_i reduced the root As concentration to approximately 30% of control values in *P. vittata* and *P. cretica* and to approximately 20% in *N. exaltata* (data not shown). Maximal inhibition was seen at 250 μM P_i in all fern species, and there was little further inhibition by increasing the P_i concentration to 500 μM. By interpolation, the concentration of P_i at which As^V uptake was reduced by 50% was approximately 30 μM, and this P_i concentration was chosen to study the effects of P_i on As^V uptake kinetics.

In the kinetic study, 30 μM P_i caused a significant inhibition of As^V uptake in both the hyperaccumulator and non-accumulator fern species, and caused the rate of As^V influx to fall to approximately 40% of control influx values for 5 μM As^V (Fig. 3). By contrast, 5 μM P_i only slightly depressed As^V influx in both fern species at all As^V concentrations used.

Phosphate had no significant effect on V_{max} values in either *P. vittata* or *N. exaltata* but, as shown in Table 1, 30 μM P_i caused a significant increase in K_m values for both species (*P. vittata* $P=0.01$, *N. exaltata* $P=0.04$), which is consistent with competitive inhibition of As^V influx by P_i. The r^2 values for the Hanes-Woolf linear plots with 30 μM P_i (not shown) were 0.83 for *P. vittata* and 0.73 for *N. exaltata*. The 5 μM treatment also increased K_m in both species, but unfortunately, the

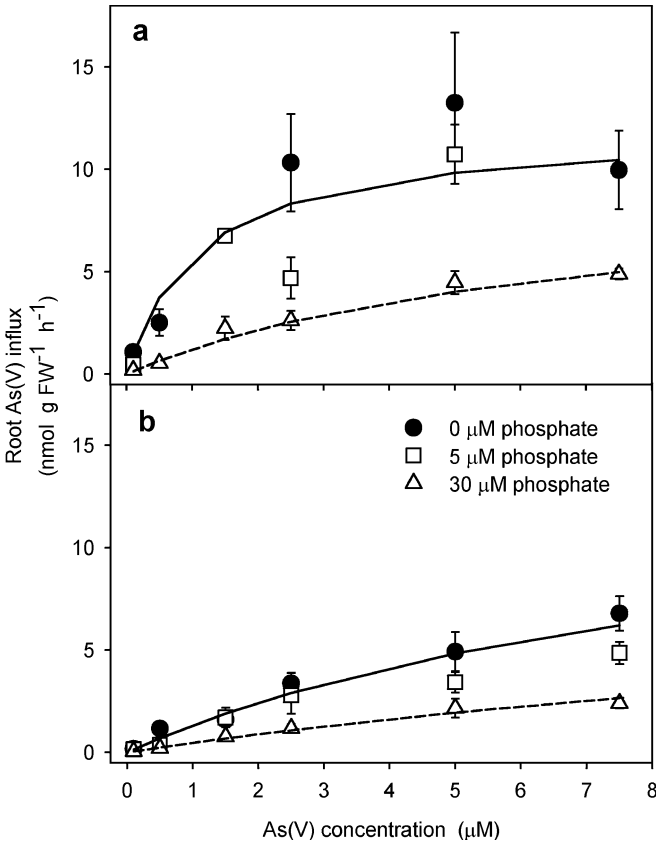

Fig. 3a, b The effect of phosphate on concentration-dependent influx of ^{73}As-labelled As^{V} into roots of (**a**) *P. vittata* and (**b**) *N. exaltata*. The influx time was 30 min. Data points and error bars represent means ± SE of 3 or 4 replicates and some error bars are smaller than the symbol. Lines are models generated by inserting the derived kinetic parameters into the Michaelis-Menten equation: *solid line*, 0 μM phosphate; *dashed line*, 30 μM phosphate (model has not been fitted to 5 μM data)

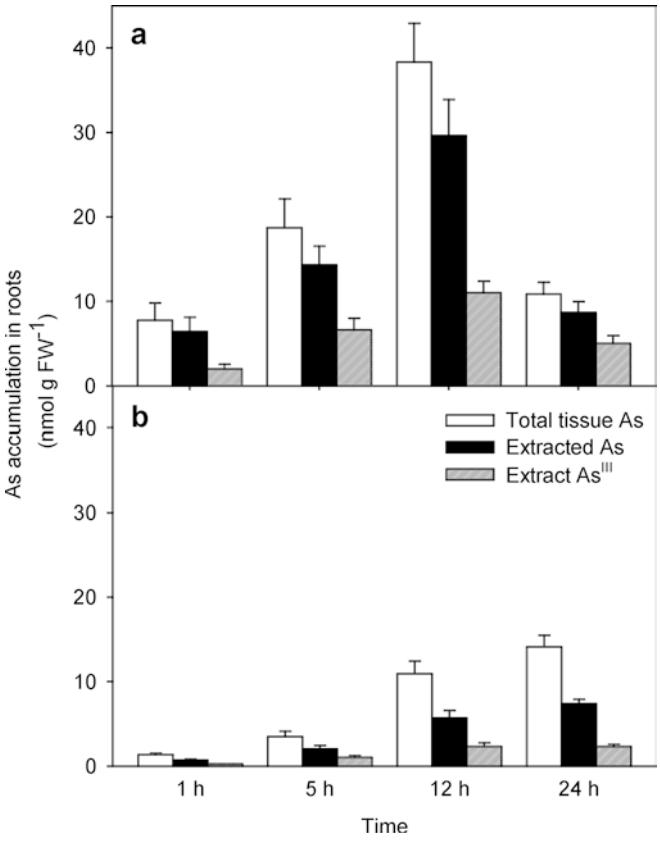

Fig. 4a, b Time course of ^{73}As accumulation in roots of *P. cretica* (**a**) and *N. exaltata* (**b**). Fractions shown are total root tissue As; 'extracted As', the As which could be extracted from the tissue by an ethanol/water extraction; and 'extract As^{III}', the amount of As^{III} present in the extracted As. All concentrations are expressed as nmol As per g FW of root tissue used. Roots were exposed to 2.7 μM ^{73}As-labelled As^{V} for the times shown. Data points and error bars represent means ± SE of 3 replicates

transformed data had a low r^2 value; therefore, the kinetic parameters have not been included in Table 1.

Arsenic accumulation and speciation in the root

Roots were allowed to accumulate ^{73}As and then the readily extractable ^{73}As fraction (which approximates the mobile pool in the root), as well as the fraction of this mobile As pool that was As^{III}, was determined. The amount of As that could be extracted from root tissue was greater in *P. cretica* than in *N. exaltata*, and in addition the extractable As made up a significantly larger *proportion* of the total tissue As in *P. cretica* (Fig. 4). Using an ethanol/water extraction with pestle and mortar, only approximately 50% of the As in the tissue could be extracted from roots of *N. exaltata*, compared with approximately 80% from *P. cretica*. Results from *P. cretica* were very similar to those obtained in *P. vittata* (data not shown) and therefore *P. cretica* was chosen to represent both As hyperaccumulator species in this experiment. The proportion of extractable As to total As varied little within each species over the 24-h duration of the experiment. A significant proportion of the As^{V} was reduced rapidly in the root to As^{III}; 35–50% of the extractable As was present as As^{III} after only 1 h (Fig. 4). The concentration of extractable As^{III} in roots and also this concentration as a proportion of the total tissue As were both significantly greater in *P. cretica* than in *N. exaltata*. After 12 h accumulation there was 11 ± 1.4 nmol extractable As^{III} g FW^{-1} in roots of *P. cretica*, or 29% of the total As, whereas in *N. exaltata* the roots contained 2.3 ± 0.5 nmol extractable As^{III} g FW^{-1}, or 21% of the total tissue As. It should be noted that the drop in root As concentration in *P. cretica* after 24 h is a result of the depletion of the As from the solution, combined with the continued translocation of As to the shoots (see also Fig. 1).

Arsenic translocation to the shoot

P. cretica showed significantly greater translocation of As to the shoot than *N. exaltata* during 24 h of uptake and transport from 2.7 μM ^{73}As-labeled As^{V} solution.

Translocation, expressed as the amount of As in the shoots as a percentage of the total amount of As in the whole plant, was 74% in *P. cretica* and 8.8% in *N. exaltata*. In both species, the addition of P_i to the uptake solution produced a small, but not statistically significant reduction in the translocation of As to the shoots, to 64% or 59% in *P. cretica* and to 6.8% or 6.5% *N. exaltata* with 5 or 30 μM P_i, respectively.

Arsenic accumulation and speciation in the shoot

Arsenic accumulated rapidly in shoots of *P. cretica* after incubation of roots in 2.7 μM ^{73}As-labeled As^V solution (Fig. 5). There was insufficient As accumulation in shoots of *N. exaltata* to determine the amount of readily extractable As or speciation of As in the shoots of this species (see Fig. 1). After 5 h of accumulation by *P. cretica*, 95% of the shoot tissue As could be extracted by ethanol/water extraction with mortar and pestle, but by 12 h, this fraction had fallen to 82% of the total (Fig. 5). Of the extractable As, the proportion of As^{III} increased over time, from 65% after 5 h accumulation to 80% after 24 h.

Discussion

There was a clear difference between the As hyperaccumulator and non-accumulator fern species with regards to the uptake and accumulation of As^V over the short time periods investigated here, with *Pteris cretica* cv. Mayii accumulating more than five times the amount of As in *Nephrolepis exaltata* (Fig. 1). This appears to be due to an increase in both rate of root As^V uptake and translocation to the shoot in the As hyperaccumulator species.

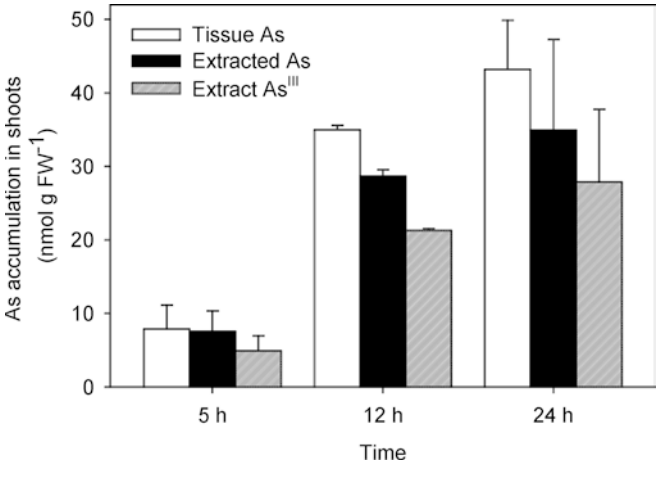

Fig. 5 Time course of ^{73}As accumulation in shoots of *P. cretica*. Fractions shown are concentrations of total shoot tissue As; 'extracted As', the As which could be extracted from the tissue by an ethanol/water extraction; and 'extract As^{III}', the concentration of As^{III} present in the extracted As. All concentrations are expressed as nmol As per g FW of shoot tissue used. Roots were exposed to 2.7 μM ^{73}As-labelled As^V for the times shown. Data points and error bars represent means ± SE of 3 replicates

Kinetics of root As influx

Root As uptake was investigated further in short-term radiolabeled flux experiments, which were employed to quantify unidirectional As influx in roots. Both *P. vittata* and *P. cretica* were found to have a significantly larger influx of As^V than *N. exaltata* over the concentration range used in this study (0.1 to 7.5 μM As; Fig. 2). In an enzyme-catalyzed reaction, as the substrate concentration increases, a greater number of active sites are filled until the maximal rate of reaction, V_{max}, is reached. At this point, all sites are filled and a further increase in substrate concentration does not lead to any increase in rate. The rate of reaction depends upon the number of enzyme molecules, their affinity for the substrate and the substrate concentration, and this relationship can be represented by the Michaelis-Menten equation. The root As^V influx in all three species showed concentration-dependence and followed Michaelis-Menten kinetics; therefore, it can be concluded that the influx is protein-mediated and As^V binds to a discrete active site, both of which would be expected for an anion such as As^V. The Michaelis constant, K_m, is the substrate concentration for half-maximal rate of reaction and the lower the K_m value, the greater the reaction rate at low substrate concentrations. The difference in root As influx rate appears to be due to a lower K_m for As^V in the hyperaccumulator species compared with *N. exaltata*. This suggests that the As^V-uptake proteins in the root cell plasma membrane of the *Pteris* species have a higher affinity for As^V. The fact that the V_{max} was found to be similar in *P. vittata* and *N. exaltata* suggests that they have similar numbers of these transport proteins per unit of root plasma membrane (assuming the same amount of plasma membrane per root mass in both species). The higher affinity in *P. vittata* results in a higher proportion of the transporters binding and transporting As^V anions at any given time, when the As influx is well below the V_{max}, which could result in a larger root As influx at lower solution As^V concentrations. When we modeled the concentration-dependent kinetics of As^V influx as a simple Michaelis-Menten function using the kinetic parameters obtained here (similar V_{max} values but 3- to 9-fold differences in K_m values) the hyperaccumulator species maintained significantly higher As influx at As concentrations below those that yielded maximal flux rates (Figs. 2,3).

The K_m for As^V uptake in *P. vittata* reported here is very similar to that found by using a solution-depletion technique to measure As^V uptake over 8 h from a solution initially containing a single concentration of 5 μM As^V (Wang et al. 2002). However, the V_{max} values determined by Wang et al. (2002) were higher than those measured in the current study, possibly due to differ-

ences in growth of plants and P_i status (Wang et al. also showed that 8 days of P_i starvation increased the rate of uptake) or to differences in the techniques used, as the depletion method measures net solute fluxes and not unidirectional influx.

Evidence that As influx is via a P_i-transport protein

The hypothesis that As^V enters roots of the As-hyperaccumulating *Pteris* species through a P_i transporter, as has been reported for non-accumulating plant species (e.g. Meharg and Macnair 1992), was supported here by the nature of the P_i-mediated inhibition of As^V uptake in both types of fern species. Phosphate has been shown to inhibit As^V uptake into *P. vittata* in both the short-term experiments in the current study (Fig. 3) and by Wang et al. (2002), who studied the effect of 50 μM P_i on As uptake using the solution-depletion method. In the current study, because we determined the effect of P_i exposure on the concentration-dependent kinetics of As^V influx, we were able to look at the interaction of P_i with As influx in more detail. As depicted in Table 1, in both species the K_m was increased by P_i exposure, with no consistent effect on V_{max}, indicating that the inhibition is competitive. This is consistent with As^V being transported across the root cell plasma membrane on a P_i-transport protein, as both molecules appear to compete directly for uptake. This demonstration, through quantitative analysis of As^V-uptake kinetic parameters, that the inhibition of As^V uptake by P_i is competitive, in both As-hyperaccumulating and non-accumulating fern species, agrees with earlier work in grasses (Meharg and Macnair 1990; Meharg et al. 1994). In addition, the K_m values for As^V transport measured here are similar to those determined for high-affinity P_i transport (1–5 μM; Kochian 2000).

The As^V concentration range used was chosen to be in the physiological range of the high-affinity P_i transporter (Kochian 2000) in order to avoid potential complications resulting from multiple transport systems acting at once (high- and low-affinity transporters), and also because the low-micromolar range is relevant to the occurrence of As in contaminated drinking and ground water. Concentrations in contaminated soils may be higher, often in the range 10 to 100 mg kg^{-1}, but only a small proportion of the total As will be dissolved in the soil solution and the amount depends on the physicochemical properties of the soil. Caution should be exercised in extrapolating results presented in physiological studies such as this to more complex soil environments.

It was surprising that the presence of an equal concentration of P_i as As^V (5 μM), did not produce a greater reduction in As^V uptake than was seen. If the transporter had an equal affinity for As and P_i, then the addition of an equal concentration of P_i should reduce As uptake by 50%. The fact that this produced only approximately 30% inhibition suggests that the transport protein has a higher affinity for As^V than for P_i. If this were the case, it would be thought that this would not be beneficial to the plant, but possibly it cannot be avoided. Arsenic-tolerant *Holcus lanatus* appears to reduce As accumulation, not by modification of the high-affinity transporter, but by down-regulating its expression (Meharg and Macnair 1992).

Based on the lower affinity for As^V transport in roots of *N. exaltata*, it might have been expected that P_i would have caused greater inhibition of As^V uptake than in the *Pteris* species, but this was not the case. This expectation is based on the assumption that both the hyperaccumulating and the non-accumulating plants studied here have a similar affinity for P_i, but different affinities for As^V. However, *Pteris* species may have a greater affinity for both As^V and P_i, rather than a selectively greater affinity for As^V over P_i.

Increased translocation and mobility of As in hyperaccumulator species

The second major difference between the As hyperaccumulator and non-accumulator species is the increased rate of transfer of As to the shoot in the As hyperaccumulator species (Fig. 1). When roots of both species were studied further, a greater fraction of the accumulated As could be extracted from the roots of *P. cretica* (in addition to a greater absolute concentration) than from roots of *N. exaltata* (Fig. 4). This suggests that there is a greater pool of mobile As in the roots of the *Pteris* species, available for translocation to the shoot. In contrast, in *N. exaltata*, a larger proportion of As may be sequestered, most likely in the vacuoles, which may not have been completely disrupted by our extraction method. This is similar to what has been suggested for Zn translocation in the Zn/Cd hyperaccumulator, *Thlaspi caerulescens*. Compared to the related non-accumulator, *Thlaspi arvense*, a smaller fraction of absorbed Zn was stored in root vacuoles of *T. caerulescens*, thereby maintaining a much larger mobile pool of Zn in the root, which was suggested to contribute to the greater translocation of Zn to the shoot (Lasat et al. 1998). In *P. cretica* after 24 h accumulation, by which time the plants had depleted the solution As^V and much of the As had already been translocated to the shoots, the fraction of *non*-extractable As in the roots had also decreased in line with the decrease in overall root As concentration. This indicates that even this 'non-extractable' fraction may be re-mobilized by *P. cretica* for transfer to the shoots. It is possible that the As hyperaccumulator species also sequester a small amount of As in root vacuoles (although less than the non-accumulator species), but that the hyperaccumulators possess a mechanism for As efflux from the vacuole to re-mobilize the As, that is absent from non-accumulator species. Again, this is analogous to Zn hyperaccumulation in *T. caerulescens*, in which efflux from root vacuoles has been found to be almost twice as rapid as in the non-accumulator *T. arvense*, thus making the Zn ab-

sorbed by the roots of the hyperaccumulator readily available for loading into the xylem (Lasat et al. 1998).

As speciation

Arsenate taken up by roots of all three species was reduced to As^{III} extremely rapidly—approximately 40% of the extractable As was found as As^{III} within 1 h—although the absolute *amount* of As^{III} was much higher in the As hyperaccumulators. This demonstrates the capability for reduction of As^{V} in the roots of both hyperaccumulator and non-accumulator species. It might be expected that the hyperaccumulators would have greater capacity to reduce As^{V} to As^{III}, especially if As^{III} is the form that is transported to the shoot (suggested by Pickering et al. 2000; Wang et al. 2002). There was a greater concentration of As^{III} in roots of *P. cretica* than in *N. exaltata*, although the amounts of As^{III} as a percentage of extractable As were similar between the two species (until the 24-h time-point). It may have been that reduction *was* faster in the hyperaccumulator, but that once reduced to As^{III}, it was rapidly moved to the shoot, thus no longer contributing to the pool of As^{III} in the root. The percentages of As^{III} in the extractable fractions of roots did diverge significantly after 24 h, with nearly 60% As^{III} in roots of *P. cretica* and slightly over 30% in roots of *N. exaltata*. There may have been a continual high rate of reduction to As^{III} over the course of the experiment in the *Pteris* species, but by 24 h, due to depletion of As from solution, no longer a significant influx of As^{V}, leading to an increase in the percentage of As^{III} in the roots. The large proportion of As^{III} found here is in contrast to the data of Ma et al. (2001), who found only 8% As^{III} in the roots of *P. vittata* grown in contaminated soil for 20 weeks, which may reflect the large difference in time-scales between the two studies. The plants in the latter study may either have adjusted to a more steady-state condition or may have had some secondary reaction to high As concentrations over an extended time. Data generated in short-term experiments, such as these, may not always be easily compared to those from long-term studies, which study aspects of As accumulation outside the scope of this work, such as the long-term effects of high tissue As accumulation.

The suggestion that As is likely to be translocated as As^{III} is consistent with the lack of significant effect of P_i on translocation of As to the shoot in the longer-term experiment (24 h) in either *P. cretica* or *N. exaltata*. An inhibition of As translocation by P_i might be expected if As were translocated as As^{V}. The translocation of As^{III} to the shoot could involve transport systems other than those for P_i. The most likely candidate for this would be aquaporins (AQPs); both yeast and mammalian AQPs have been shown to mediate transmembrane As^{III} transport (Wysocki et al. 2001; Liu et al. 2002) and recent evidence indicates the same may be true in rice (Meharg and Jardine 2003).

The proportion of the extractable As fraction present as As^{III} in the shoots of *P. cretica* increased from 63% after 5 h of root exposure to As^{V} to 80% after 24 h (Fig. 5). There are two possible explanations for this. Firstly, it may be that some of the As does arrive in the shoot as As^{V}, and further reduction occurs in the shoot, and secondly, As^{III} may be preferentially transported from the root. Any As^{V} that is not reduced in the root could be translocated through a P_i-pathway, but a significant fraction of the As^{V} taken up is reduced to As^{III} in the root and the translocation pathway for As^{III} is likely to be faster than for As^{V}.

After 5 h of accumulation, 95% of the As that had accumulated in shoots of *P. cretica* could be extracted, indicating that the As in the shoot was still in a mobile pool. By 12 h, it seems that sequestration had started to occur, as this proportion had fallen to 82% (Fig. 5).

Conclusions and future directions

To date, much of the research into the As hyperaccumulation of *Pteris vittata*, and other species in the *Pteris* genus, has focused on the accumulation in the above-ground organs. This is where the ultimate accumulation of As occurs in these species, and fronds are also much more accessible for study than roots. However, evidence is starting to emerge that key mechanisms for the hyperaccumulator phenotype also reside in the roots. Here we have demonstrated that As^{V} influx into the roots of both *Pteris vittata* and *P. cretica* is greater than in a non-accumulating fern, *Nephrolepis exaltata*. We have also shown that this may be due to a higher affinity of the transport protein responsible for As^{V} influx in the hyperaccumulator species, and that this protein is almost certainly a (high affinity) P_i transporter. The higher rate of As^{V} entry into hyperaccumulator roots certainly contributes to the significantly greater translocation of As to the shoots. Furthermore, another contributing factor to this enhanced As translocation is the decreased sequestration of As in the roots observed here, in comparison with non-accumulator species. This results in a larger pool of mobile As (predominantly as As^{III}) available for transport to the shoot.

While detoxification of As in the shoots is undoubtedly important in As hyperaccumulation (probably by complexation with thiol-containing compounds such as phytochelatins, although this is still the subject of much debate; Hartley-Whitaker et al. 2001; Zhao et al. 2003), the shoots are unlikely to exert much control over the amount of As that they receive. A key question of As hyperaccumulation, therefore, is not only how does *Pteris vittata* deal with so much As in the shoot, but why is that much As there in the first place? This work begins to answer that question, and further research should be directed at the below-ground portions of these fascinating plants.

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